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EFFECTS OF EXTREMELY LOW FREQUENCY ELECTRO-
MAGNETIC FIELDS ON GROWTH AND DIFFERENTIATION
OF 'PHYSARUM POLYCEPHALUM'

E. M. Goodman, et al

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Growth and Differentiation of Physarum polycephalum

Technical Report

Phase I

(Continuous Wave)

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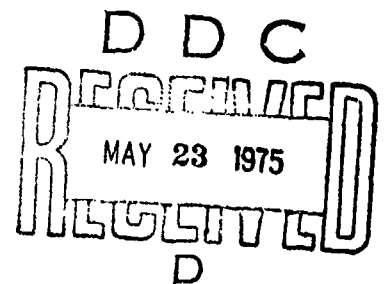
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ABSTRACT

Microplasmodia from the slime mold Physarum polycephalum have been continuously exposed to weak electromagnetic fields at 60 and 75 Hz. To date, microplasmodia have been exposed to fields of 75 Hz, 2.0 G, 0.7 V/m for more than 700 days. Another set of cultures has been exposed to 60 Hz, 2.0 G, 0.7 V/m for more than 400 days. The time between successive mitotic divisions in cultures exposed to these fields varied from 0.5 to 2 hours longer than their respective controls. This delay was discernable after approximately 90 to 120 days of exposure to electromagnetic radiation. The ability to complete both the sexual (sporulation) or asexual (spherulation) life cycles was not affected but a retardation in reversible protoplasmic streaming was observed.

INTRODUCTION

Virtually all living organisms are exposed continuously to naturally occurring, extremely low frequency electromagnetic radiation. Although it has been established that organisms both sense and respond to this type of radiation, detailed information related to the biological processes affected by these low frequency fields is limited (1-3). This article describes a series of experiments on the effects of extremely low frequency electromagnetic fields (ELF EMF) that have been conducted over the past three years on cultures of Physarum polycephalum. Since the nature and extent of ELF EMF effects in Physarum could not be anticipated, our experiments were designed to monitor a wide range of biological processes for extended periods of time. We observed alterations in the timing of the cell cycle and shuttle streaming of Physarum that have been ascribed to ELF EMF exposure.

The myxomycete Physarum polycephalum was selected as the experimental organism for several reasons: 1) it is easily maintained as microplasmodia in submerged shake flask culture, 2) the various phases in its life cycle can be readily induced at the discretion of the investigator, 3) if growing microplasmodia are allowed to coalesce on filter paper a series of naturally synchronous mitoses will occur at predictable intervals, and 4) extensive biochemical and physiological data on Physarum are already available (4-6).

In the interest of completeness both null-result experiments as well as those displaying significant alterations have been described briefly. We wish to emphasize that because of the survey nature of this study the most sensitive assays and techniques available were not always selected.

MATERIALS AND METHODS

Maintenance of Cultures and Mitosis Experiments: Submerged shake flask cultures of Physarum polycephalum (line M₃C VI) were obtained from Dr. J. Mohlerg, McArdle Cancer Laboratory, Madison, Wisconsin. Our experiments were divided into three

general areas: 1) vegetative growth, 2) the asexual life cycle, and 3) the sexual life cycle. Separate control and experimental cultures were designated for each area. Cultures were grown in the semi-defined medium of Daniel et al. (7) using specially designed flasks (See Section II). For initial experiments on vegetative growth, four sets of cultures were designated control cultures to which no electric or magnetic fields were applied; four others were designated experimental cultures and subjected to electromagnetic fields of 2.0 G and 0.7 V/m at 75 Hz¹. Control cultures served as the inoculum source for all subsequent experiments involving different field intensities. Submerged cultures were grown on reciprocal shakers (New Brunswick, R-7, New Brunswick, N. J.) operating at 122 strokes per minute. Control and experimental cultures were maintained in separate incubators (Warren-Sherer, RI-48, Marshall, Michigan) at $25.5 \pm 0.3^{\circ}\text{C}$. Incubator modifications are described in Section II.

Cell Cycle: The time required for macroplasmodia to attain the second metaphase configuration was determined. Stationary macroplasmodia were prepared from submerged microplasmodia according to the procedures of Mittermayer et al. (8) with two modifications: 1) petri dish tops were altered to accommodate stainless steel electrodes and 2) two layers of Rapid-Flo gauze (6.5 cm x 6.5 cm, Filter Fabrics, Goshon, Ind.) replaced the stainless steel grids commonly used as supports for the filter paper.

Each experiment consisted of 10 control plates (derived from cultures in the control environment) and 10 experimental plates (derived from cultures that were continuously subjected to the particular field condition under investigation). Stages in the mitotic cycle were ascertained by observing ethanol-fixed smears under a phase contrast microscope. Following the addition of medium, control cultures

1 The experimental fields in this study were approximately 10 x greater than the .26 G, 0.7 V/m projected for the vicinity of the proposed project Sanguine.

generally required 14.5 to 15 hours to reach metaphase of the second mitotic division. Occasionally, the time required to attain the third metaphase configuration was also determined.

Vegetative encystment: Two parameters were examined in these experiments: the competence of microplasmodia to form microsclerotia (spherules) and their ability to germinate afterwards. Spherulation was induced by transferring microplasmodia into a non-nutrient salt solution (7, 9). To induce germination microsclerotia were washed twice in distilled water to remove excess polysaccharide and then inoculated to nutrient media. The competence of plasmodia to form spherules or to germinate was quantified by the pigment extraction technique of McCormick et al. (10). This procedure is based on an inverse relationship between encysted plasmodia and extractable pigment. Confirmation of plasmodial status was also obtained by visual observation with phase optics.

Sexual Cycle: Three parameters were studied in these experiments: 1) the competence of microplasmodia to sporulate, 2) the ability of these spores to germinate, and 3) the fusion of haploid myxamoebae to reform plasmodia. Microplasmodia were induced to sporulate according to the procedures of Daniel et al. (7). Spore germination and the fusion of myxamoebae were studied using procedures developed by Dr. J. Mohberg (personal communication). Spores were liberated by breaking sporangia in a Potter-Elvehjem homogenizer followed by several washes in distilled water before inoculation to 1/10 strength growth medium in 4% agar. Spores germinate within 12 to 24 hours forming haploid myxamoebae that fuse to reform the diploid plasmodial or vegetative stage. Newly formed plasmodia were removed aseptically from the agar, retransferred to submerged growth conditions, and the cycle repeated.

Protoplasmic Streaming: The shuttle streaming period of control and experimental cultures was monitored to ascertain if exposure to ELF EMF induced deviations from normal streaming patterns (11). Submerged microplasmodia were selected in log phase of their growth cycle, a streaming channel chosen, and the time required to

complete one oscillatory period determined. A minimum of four determinations were made on each channel.

Proteins: Growth rates in submerged shake cultures were measured by determining the protein content in both control and experimental lines as a function of time. Duplicate samples from both control and experimental submerged growth cultures were removed at periodic intervals, centrifuged at $250 \times g$ (4°C) for 10 minutes and the supernatant decanted. Yellow pigment was removed by repeated extractions with trichloroacetic acid-acetone (5%/50% v/v) (7) and the residual pellet was dissolved in 0.4 N NaOH. Protein content was determined according to the procedure of Lowry et al. (12) using bovine serum albumin as a standard.

Nucleic Acids: The synthetic profile of DNA replication was determined by subjecting duplicate samples from both control and experimental cultures to a 10 minute pulse of ^3H thymidine ($0.6 \mu\text{C}/\text{ml}$, Sp. Act, $1.3\text{mC}/\text{mm}$, Schwarz/Mann, Orangeburg, New York). The first pulse was administered at approximately telophase of the second mitotic division and every 30 minutes thereafter over a four hour period. Following each radioactive pulse, plasmodia were placed on growth medium (without isotope) for twenty minutes. At the end of this chase period DNA was extracted according to the procedure of Sachsenmaier and Rusch (13). Samples were counted in Bray's solution (14) using a Unilux 11 A scintillation spectrometer with counts corrected to 100% efficiency using the Nuclear Chicago CURFIT program. The total protein in the sample was determined as described above.

RNA profiles in control and experimental cultures were followed between the second and third mitotic divisions using ^3H uridine ($2.5\mu\text{C}/\text{ml}$, Sp Act. $2.0\text{C}/\text{mm}$, Schwarz/Mann, Orangeburg, New York). Plasmodia were pulsed for 15 minutes, beginning at telophase and at 30 minute intervals until prophase of the next mitotic division. The procedures of Sachsenmaier and Rusch (13) were used to extract RNA, while radioactivity and protein analysis followed the procedures previously described.

Field Generation and Monitoring: Magnetic and electric fields were generated using facilities and equipment described in Section II. Electrodes in all growth flasks and petri dishes in both the control and experimental incubators were wired in identical fashion; circuits were not energized in the control incubator. Temperature, current, and voltage were monitored continuously in all incubators.

RESULTS

Vegetative Plasmodia (75 Hz): Experimental cultures have been continuously exposed to extremely low frequency electromagnetic fields (ELF EMF) of 75 Hz, 2.0 G, and 0.7 V/m for more than 700 days. The data (Fig. 1a) indicate that a significant increase in the cell cycle of experimental cultures occurred after approximately 120 days of continuous exposure. Cycle differences in the experimental line initially ranged from 0.5 hours to 2.5 hours greater than the control line. These effects continued through day 250 and then appear to have dissipated. Scrutiny of our data revealed that the cell cycle in ELF EMF treated cultures had not decreased, but rather, that the cell cycle in control cultures had increased. This change in the control cycle accounts for the apparent disappearance of the electromagnetic effect. Concomitant with the change in the control cultures' cell cycle they also became excessively slimy, with macroplasmodia tending to fragment rather than maintaining their normal concentric appearance. These conditions persisted regardless of the age of microplasmodia used to prepare stationary cultures ruling out the obvious possibility that cultures selected for experimentation were in the stationary phase rather than the logarithmic phase of their growth cycle. Since these abnormal physiological and morphological changes could not be reversed a new control line was initiated on day 300 using subcultures maintained from the beginning as backup controls. The introduction of a new control line with a normal cell cycle (about 15 hrs. from inoculation to second mitosis) resulted in the reappearance of the experimental cell cycle delay.

To preclude the possibility that the change in the experimental cell cycle was a fortuitous event or related to difficulties experienced with the controls, a new set of control subcultures was transferred to the incubator operating at 75 Hz, 2.0 G, 0.7 V/m. The data (Fig. 1b) show that an increase in the cell cycle becomes significant after approximately 120 days of continuous exposure. These experiments were performed subsequent to difficulties encountered with the original control cultures.

In a search for threshold levels, cultures were transferred from the control environment to fields approximately five-fold weaker (75 Hz, 0.4 G, 0.15 V/m). No significant alterations in the mitotic cell cycle were observed after 180 days of continuous exposure to these fields (Fig 2a). In another experiment (data not shown) cultures exposed to field intensities forty times weaker (75 Hz, .05 G, 0.017 V/m) for 180 days also failed to reveal significant alterations in the timing of the cell cycle.

If cultures displaying an altered cell cycle were transferred to the control environment, the delay slowly dissipated over 30 to 50 days. In a corollary experiment, plasmodia displaying a delayed cell cycle as a result of exposure to 75 Hz fields of 2.0 G and 0.7 V/m were transferred to lowered field conditions. Continuous exposure to the weaker fields resulted in dissipation of the cell cycle delay after approximately 40 days (Fig. 2b).

Attempts were made to relate the longer cell cycle of experimental cultures to slower growth rates by comparing the protein content of control cultures with those exposed to 75 Hz, 2.0 G, 0.7 V/m. Statistically significant differences between the two sets of cultures were not evident. DNA and RNA profiles were also examined to ascertain if the cell cycle changes induced by exposure to ELF EMF could be related to alterations in nucleic acid syntheses. No significant differences in synthetic patterns was observed between control cultures and cultures exposed to 75 Hz, 2.0 G, 0.7 V/m for 114 days.

Vegetative Plasmodia (60 Hz): A second set of experiments at the same field intensities (2.0 G, 0.7 V/m) but at a different frequency (60 Hz) was initiated and continued for 360 days. The data, (Fig. 3a) indicate an increase in the mitotic cell cycle was induced, occurring earlier in the 60 Hz environment than in the 75 Hz field. To confirm these observations another set of cultures from the control environment was introduced into the experimental field (60 Hz, 2.0 G, 0.7 V/m). The data (Fig. 3b) support the suggestion that the length of the cell cycle is affected earlier in a 60 Hz ELF EMF environment.

Vegetative Encystment (spherulation) and the Sexual Life Cycle (sporulation): Continuous exposure to 75 Hz, 2.0 G, 0.7 V/m had no significant effect on the competence of microplasmodia to form spherules or on their ability to germinate. Although competence was not affected cultures repeatedly sequenced through the asexual life cycle do display a delay in their cell cycle (Fig. 4a). Similarly, long term exposure to ELF EMF does not affect the competence of microplasmodia to transverse the sexual life cycle, although continuous exposure did result in an increased mitotic cell cycle (Fig 4b). The sexual line required more than 200 days to produce a statistically significant delay in the cell cycle in contrast to the asexual and vegetative cultures which displayed a significant effect after 120 days of continuous exposure to the same field intensities.

Vegetative Encystment (60 Hz): In studies of vegetative encystment at 60 Hz, 2.0 G, 0.7 V/m, our data indicate that both competence to form spherules and their ability to germinate are not affected by continual exposure to these fields. The timing of the cell cycle was altered after a relatively short period of exposure to the 60 Hz fields (Fig. 5), substantiating the trend observed with the vegetative lines exposed to 60 Hz fields.

Shuttle Streaming: Another effect related to ELF EMF exposure is an increase in the shuttle streaming period. In contrast to the cell cycle changes the streaming effect is discernable after short-term exposure and appears to be independent of both

frequency and field strength. Streaming data were analyzed by means of a standard two-factor analysis of variance where fields are treated as a fixed factor having two levels (no field or control and a single field level). Day of observation is the second factor which is treated as a random factor having many levels. Results of this analysis are presented in Table I.

Significant field effects (the quantity of interest) were obtained in three of the four lines tested. In general, protoplasmic streaming was slower in cultures exposed to various electromagnetic fields, although, the reverse occurred on some days. The analysis reveals that a large source of variation is the day on which observations were made; also all lines studied exhibit a significant interaction between fields and days. The simple effect due to fields may be examined by treating each day's observations as paired, taking the difference between day means, and averaging over all days. These data are shown in Table II.

Related Experiments: Several experiments were also performed to investigate the possibility that the ELF EMF effects on the cell cycle and protoplasmic streaming were the result of either pH changes or electrolysis products in the medium. Elimination of pH changes as a possible variable was accomplished by measuring the pH of the medium from both control and experimental cultures (at various field intensities) at the time microplasmodia were transferred to new media and at the end of their growth period. No significant deviations between the two sets of cultures were observed. To determine if the ELF EMF effects we observed were a result electrolysis products, the medium was exposed to both control and experimental field conditions for 43 hours prior to inoculation with microplasmodia. Cultures from the control incubator were then inoculated to these pretreated media and all flasks were returned to the control environment. Comparing the cell cycle and streaming data from these cultures with the controls failed to reveal significant differences.

To investigate whether delayed fusion of microplasmodia could explain the altered cell cycle, experiments were performed to determine if differences observed at the

second mitotic division persisted at the third mitotic division. The rationale for this experiment is based on the assumption that a delay in microplasmodial fusion should result in constant differences between the control and experimental cultures at both the second and third divisions, whereas increased differences would imply that other factors are involved. These experiments showed that the mitotic delay in the experimental cell cycle increased at the third mitotic division.

DISCUSSION

Numerous studies have described the effects of both induced and natural electromagnetic fields on living systems (1-3, 16). This study differs from many of these investigations in two fundamental ways: first, alternating fields rather than static fields were employed, and second, each experimental protocol was based on continuous long term exposures of at least six months. During an exposure period of six months, plasmodia proceed through their cell cycle approximately 540 times. Based on more than 200 experiments conducted over a period of 30 months, the data show that weak, alternating electromagnetic fields (60 or 75 Hz, 2.0 G, 0.7 V/m) affect the cell cycle of Physarum polycephalum by increasing the interval between successive mitotic divisions. Data supporting this claim are presented in Figs. 1 and 3. These data were analyzed by means of a Wilcoxon signed ranks test (15) which tests the null hypotheses that experimental and control populations are identical. For the data presented in Figs. 1 and 3, this hypothesis may be rejected with at least 95% confidence ($p < .05$). In order to simplify and highlight the observed effect, the data may be pooled into 25-day groups — this has been done for data from Fig. 1a and is displayed in Fig. 6. The increase in mitosis time for experimental cultures is readily apparent. This method of displaying the data prompts one to suggest that mitosis time differences may be cyclic in nature with a period of about six months.

The cell cycle delay is reproducible and since it is induced 30% to 50% faster at 60 Hz than at 75 Hz, it appears to be frequency dependent. Once induced, a trend toward an increased mitotic delay may be inferred from the data on cultures exposed to 75 Hz, 2.0 G, 0.7 V/m for more than 300 days (Fig 1). Cultures exposed to the same field intensities (2.0 G, 0.7 V/m) but a different frequency (60 Hz) failed to display this trend. In fact the data suggest that the effect on the cell cycle has either been mitigated or alternatively that the culture may be in the trough of a cyclic period (16). At this time we are unable to confirm either explanation since these experiments were terminated by factors beyond our control.

Introduction of cultures already displaying a cell cycle delay into either a control environment (no applied fields) or into a field approximately five times weaker (75 Hz, 0.4 G, 0.15 V/m) results in disappearance of the delay in approximately 40 days. Signed ranks tests of data in Figs. 2a and 2b do not permit rejection of the null hypothesis at the 95% confidence level. Since weaker fields were unable to induce a delay in new cultures introduced into the field, it appears that threshold intensities for affecting mitosis in Physarum probably lies somewhere between these field levels.

The competence of Physarum to traverse its sexual and asexual life cycles was not affected by continuous exposure to any of the electromagnetic fields employed in this study. The data do show, however, a delayed cell cycle for cultures that were induced to differentiate continuously. The Wilcoxon test (15) applied to mitosis data in Figs. 4 and 5 permits rejection of the null hypothesis for asexually differentiating cultures at $p < .05$ (Figs. 4a and 5) and for sexually differentiating cultures at $p < .15$ (Fig. 4b).

Exposure to low frequency electromagnetic fields appears to retard shuttle streaming in Physarum. In general, the rate of streaming in exposed cultures was slower than in controls, however there were several days in the test period on which the reverse was also observed. When these data are subjected to statistical analysis, field effects significant at $p < .05$ are found in three of the four exposed

cultures. The sporadic delay reversal results in a large interaction between field and day effects and thus complicates interpretation of simple field effects. At this time the lack of a significant field effect in the first set of cultures in Table I must be attributed to the presence of a large interaction term. The existence of a large interaction term in the two-way analysis of variance suggests that an uncontrolled factor may be present in the experiments. We are presently reexamining our experimental protocol for such a factor with the hope that day-to-day variability in these data may be eliminated. Our studies indicate that shuttle streaming may be a more sensitive indicator of field effects than mitosis times, in that alterations in the streaming period seem to occur four to eight weeks before significant deviations in cell cycle are noted.

In summary, exposing the myxomycete *Physarum polycephalum* to extremely low frequency electromagnetic fields (60 or 75 Hz, 2.0 G, 0.7 V/m) for approximately 90 days (>200 mitotic divisions) increases the time required to traverse the mitotic cell cycle. If cultures displaying these effects are removed from the electromagnetic environment, the cell cycle returns to control levels in approximately 40 days (>100 divisions).

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TABLE I

Two factor analysis of variance for shuttle streaming.*

Field Conditions: 75 Hz/2.0 G/0.7 V/m

Source	SS	df	MS	F
Fields	2450	1	2450	2.01
Days	38910	23	1692	69.0 **
FXD	27969	23	1216	49.6 **
Within Cell	4858	198	24.54	

Field Conditions: 75 Hz/2.0 G/0.7 V/m (Reproducibility check)

Fields	4352	1	4352	5.42 **
Days	30513	22	1387	87.2 **
FXD	17675	22	803.4	50.5 **
Within Cell	3086	194	15.91	

Field Conditions: 60 Hz/2.0 G/0.7 V/m

Fields	8422	1	8422	10.6 **
Days	30659	25	1226	73.5 **
FXD	19812	25	792.5	47.5 **
Within Cell	3769	226	16.68	

Field Conditions: 60 Hz/2.0 G/0.7 V/m (Reproducibility check)

Fields	5321	1	5321	8.31 **
Days	72911	25	2916	224 **
FXD	16007	25	640.3	49.1 **
Within Cell	2962	227	13.05	

* = Field level and is treated as a fixed factor having two levels: control (zero field) and applied ELF EMF.

D = day of observation, a random factor having many levels

FXD = interaction term between fields and days

df = degrees of freedom

SS = sum of squares

MS = mean square

F = F-statistic

** = Significant at $p < .05$.

TABLE II

Day-average of streaming periods treated as paired observation.*

Field Conditions	Average difference in periods (sec.)
75 Hz, 2.0 G, 0.7 V/m	5.5
75 Hz, 2.0 G, 0.7 V/m (reproducibility check)	10.1 **
60 Hz, 2.0 G, 0.7 V/m	14.0 **
60 Hz, 2.0 G, 0.7 V/m (reproducibility check)	8.3 **

*For a given day the mean streaming period for controls is subtracted from the mean streaming period for exposed culture. The table entry is the average difference observed.

**Significant at $p < .05$.

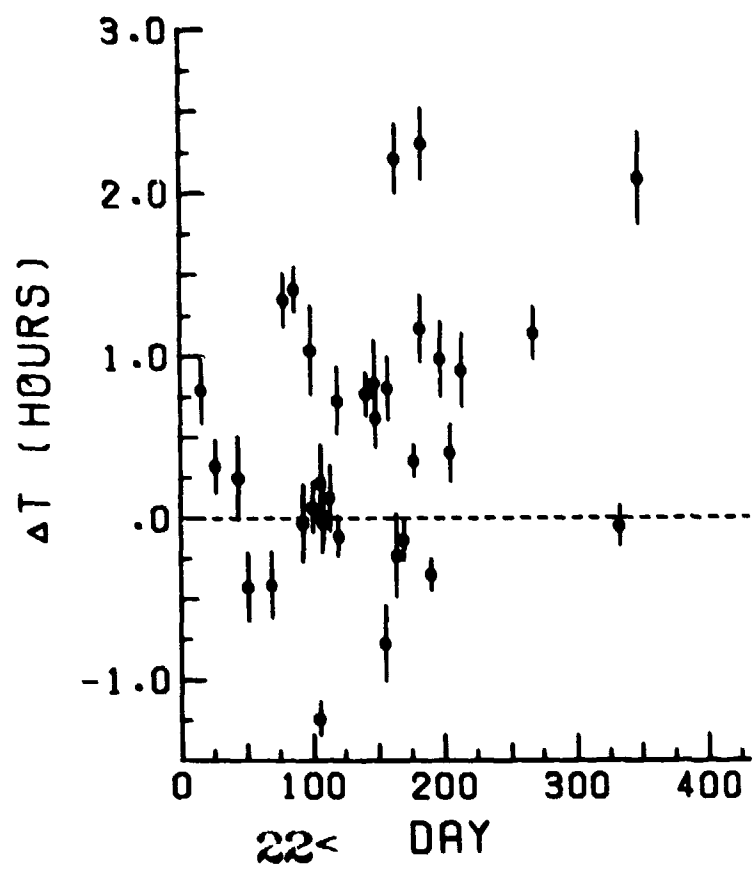
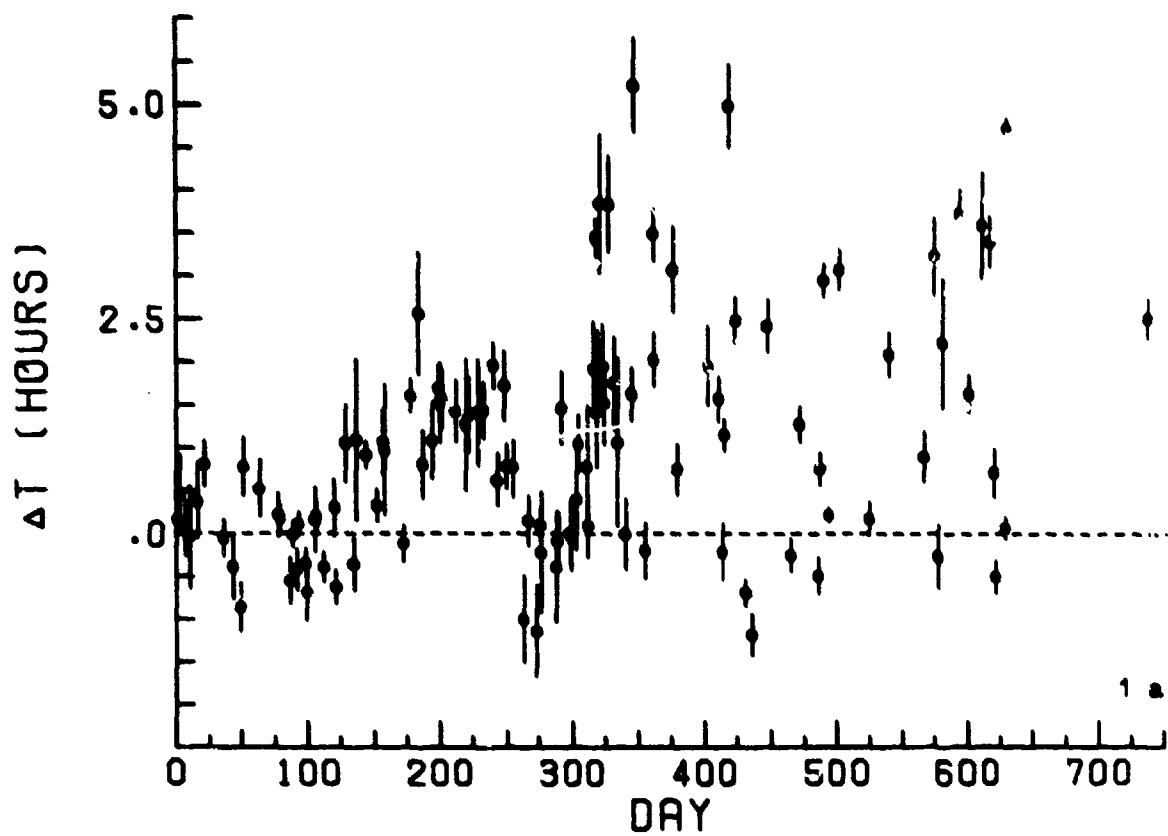
FIGURE CAPTIONS

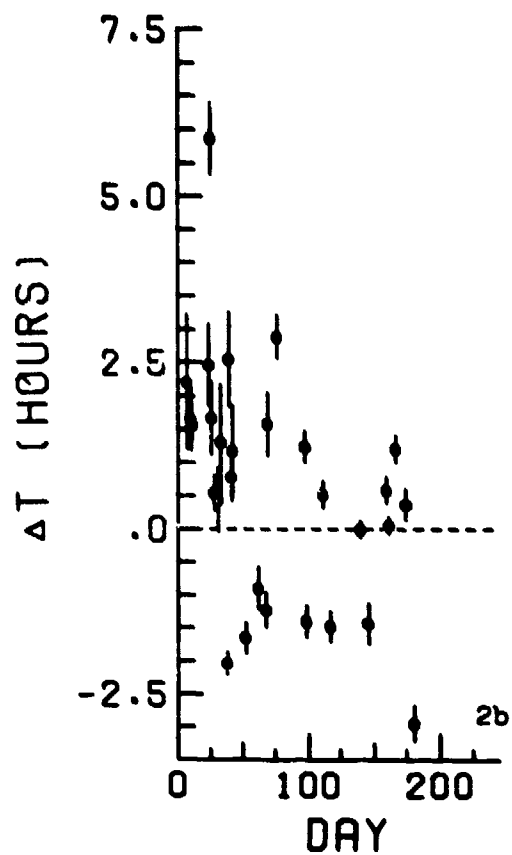
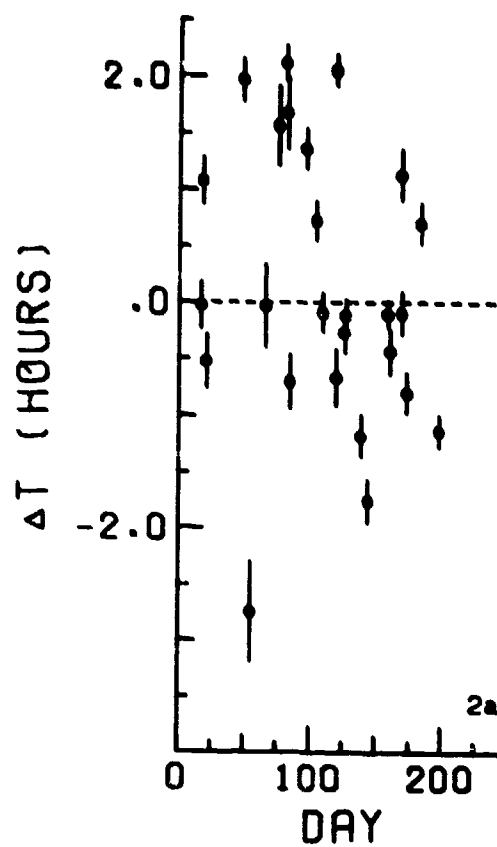
- 1 (a) Time relative to control for stationary cultures of Physarum to reach the metaphase configuration of mitosis versus number of days of continuous exposure to 75 Hz, 2.0 G, 0.7 V/m. Each point represents the average of the times for 10 experimental cultures from which the average time of 10 control cultures for that day have been subtracted. The error bars are 95% confidence limits computed by using a pooled estimate of the common variance (19).
- 1 (b) The result of introducing a new set of cultures into a 75 Hz, 2.0 G, 0.7 V/m environment to ascertain reproducibility of the cell cycle delay.
- 2 (a) Relative time to reach metaphase versus number of days of exposure to 75 Hz, 0.4 G, 0.15 V/m fields.
- 2 (b) The result of transferring a culture previously exposed to 75 Hz, 2.0 G, 0.7 V/m and displaying a mitotic delay into a weaker electromagnetic field of 75 Hz, 0.4 G, 0.15 V/m. The cell cycle delay is no longer statistically significant after about 40 days in the weaker environment.
- 3 (a) Relative time to reach metaphase versus number of days exposed to 60 Hz, 2.0 G, 0.7 V/m fields.
- 3 (b) The results of introducing a new set of cultures into a 60 Hz, 2.0 G, 0.7 V/m environment to ascertain reproducibility of the cell cycle delay.
- 4 (a) Relative time to reach metaphase versus number of days exposure to 75 Hz, 2.0 G, 0.7 V/m for cultures which have been repeatedly sequenced through the encysted and vegetative stages.
- 4 (b) Effects of a similar environment on cultures repeatedly sequenced through the sexual phases of their life cycle (vegetative plasmodia → spores → haploid myxamoebae).
- 5 Relative time to reach metaphase versus number of days exposure to 60 Hz, 2.0 G, 0.7 V/m for cultures which have been continuously sequenced through the encysted and vegetative stages of its life cycle (spherulation).

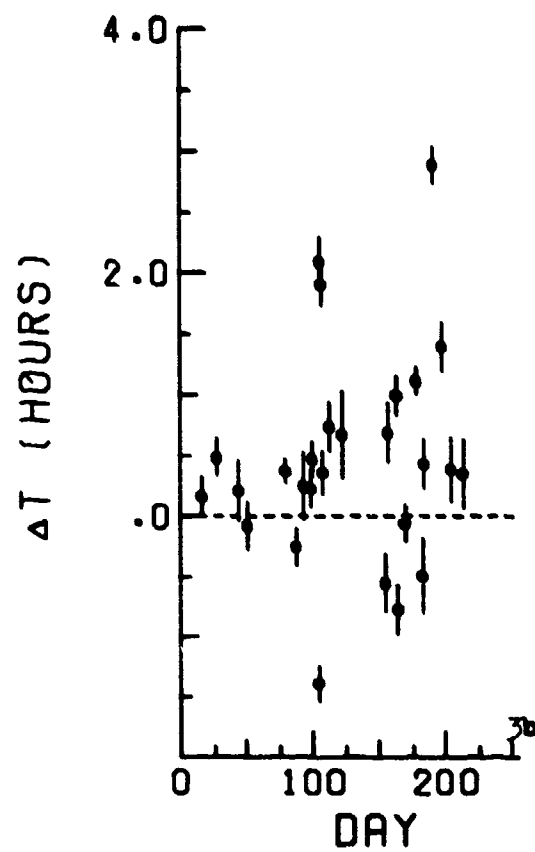
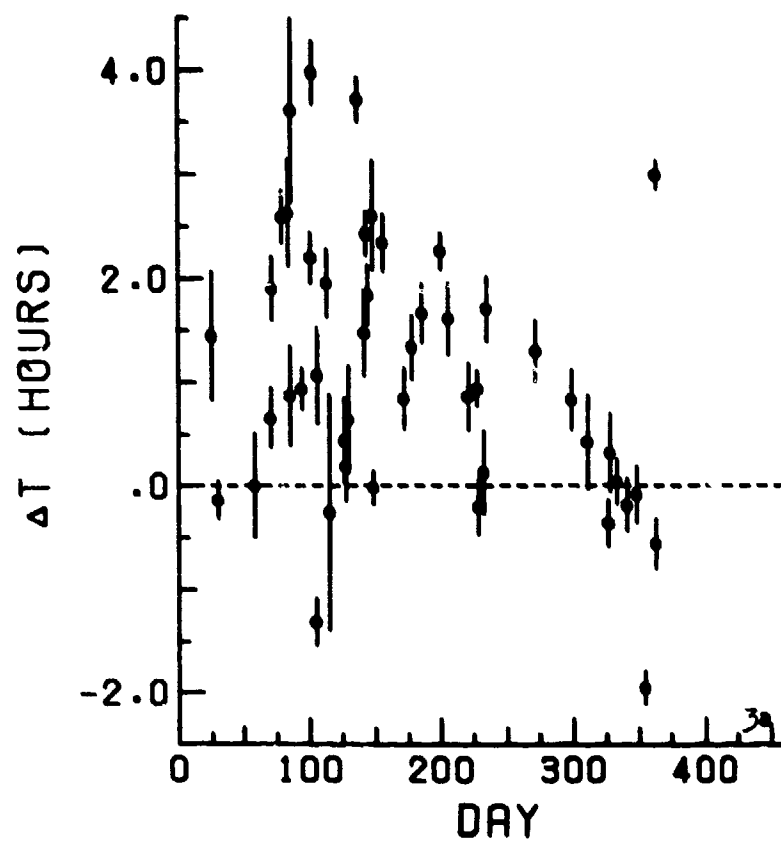
(Figure Captions - continued)

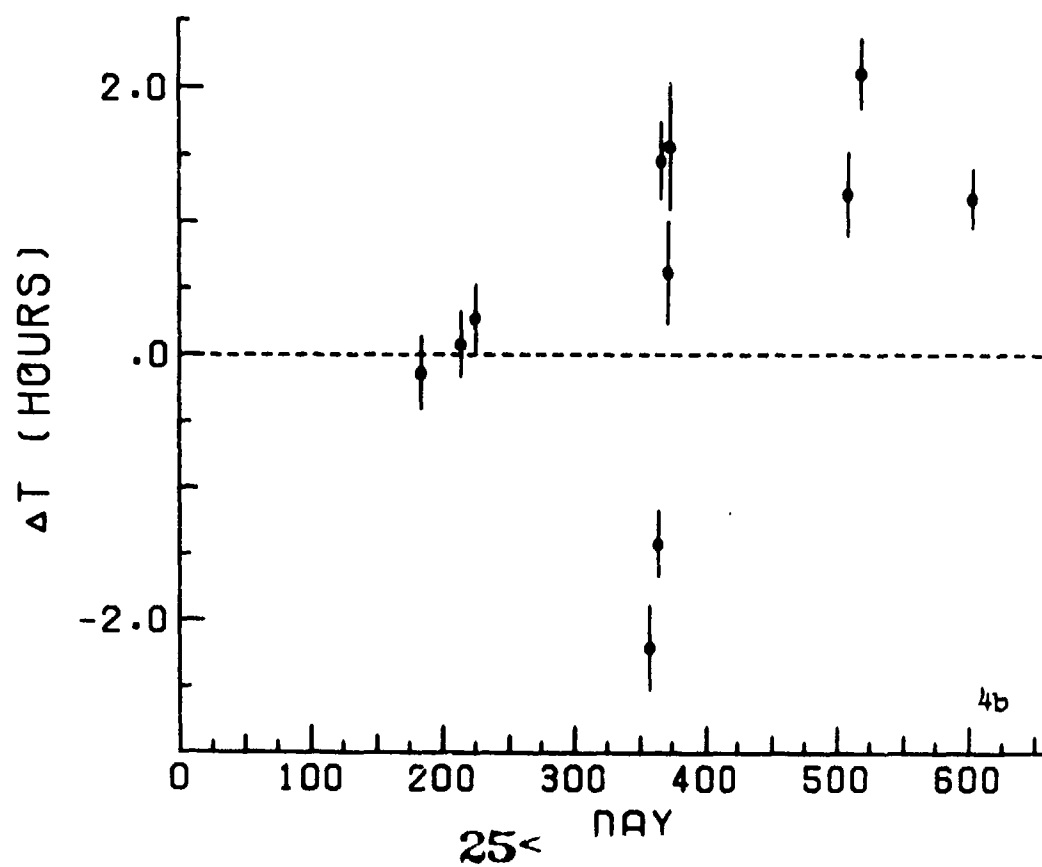
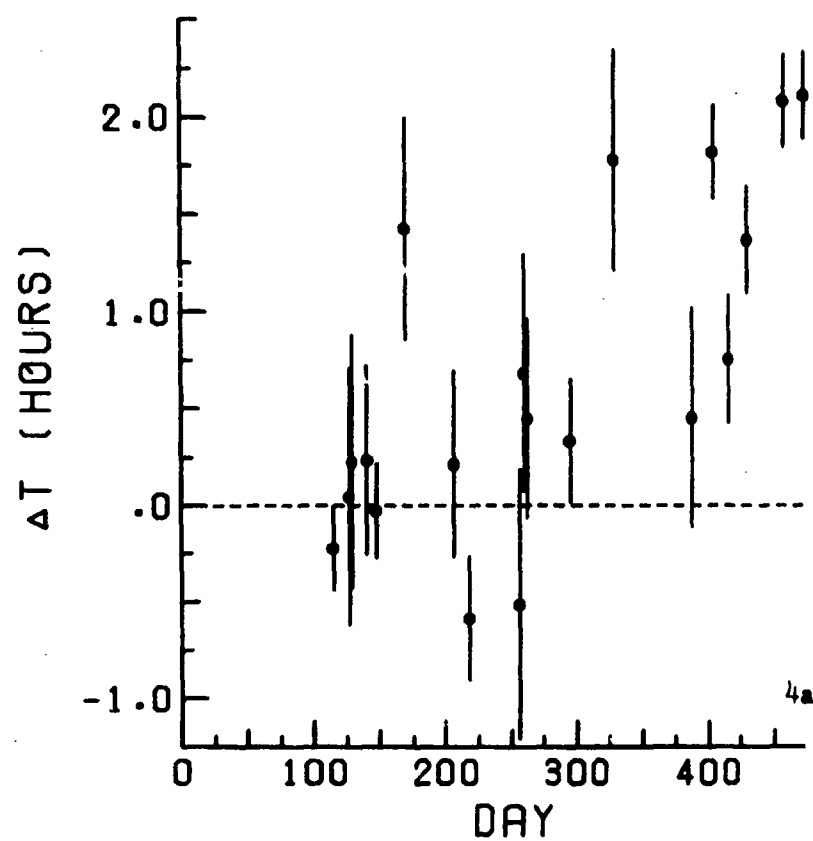
- 6 Relative time to reach mitosis versus number of days exposure to 75 Hz, 2.0 G, 0.7 V/m fields. The data from Fig. 1a has been pooled into 25 day groups.

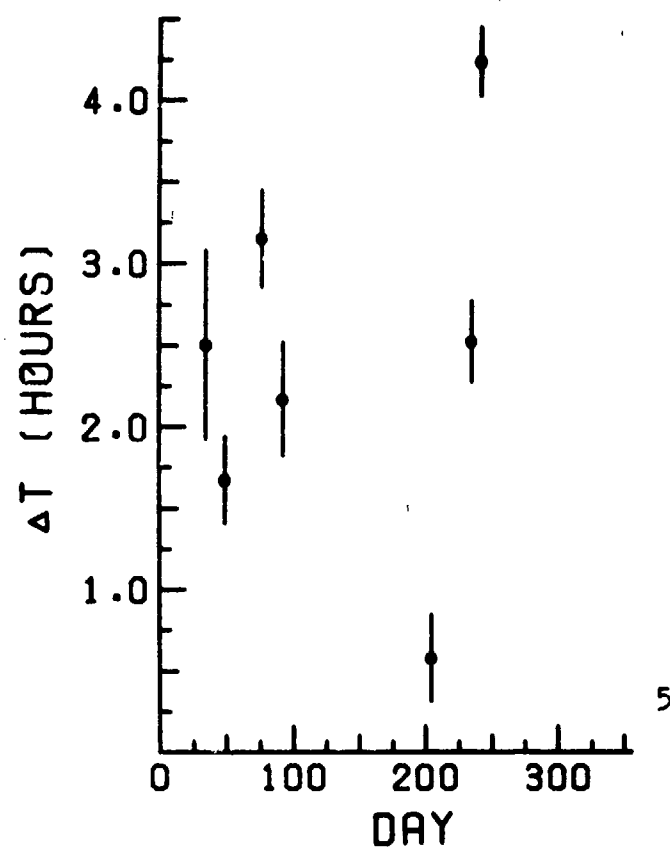
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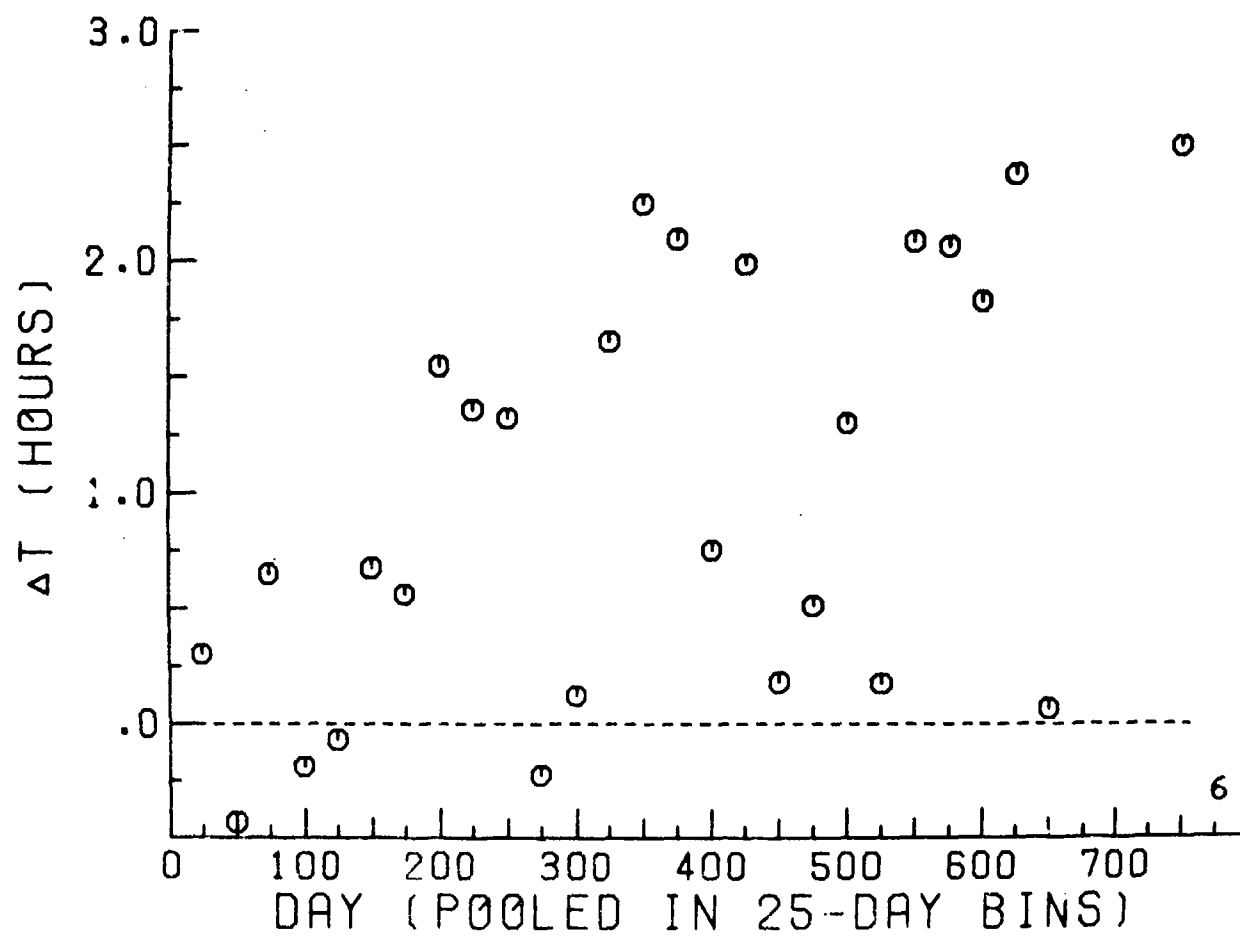












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ELECTROMAGNETIC FIELD GENERATION AND ASSOCIATED APPARATUS

I. Introduction

The preceding sections present the chief features, parameters of interest and results of the experiments. In designing the experiments we required that both the stock cultures and the macroplasmodia were to be kept continually in the fields. To isolate sets of cultures being subjected to different field conditions, each set was to be kept inside a separate constant temperature incubator. Four incubators were used. One contained the control cultures, which were not subjected to any applied fields, and the others had various experimental field conditions maintained inside them, as outlined above. The temperatures and the interior fittings of all incubators, including the one housing the control cultures, were identical.

In the following sections we outline our methods for (1) modifying the incubators to reduce sources of possible systematic errors, (2) applying the magnetic fields, (3) applying the electric fields, (4) generating and monitoring the necessary electrical signals to excite the fields, and (5) attending to certain other needed details of the experiment.

II. Incubators

The four incubators were standard Warren-Sherer Model RI-48 units. The units had a few minor differences, since one was delivered in 1970, one in 1971, and two in 1972. They have interior dimensions of 136 cm wide x 66 cm deep x 145 cm high. Two doors in the long side furnish access. We made several modifications to provide a good electromagnetic background environment for the experiments. The standard fans supplied to circulate air inside the incubators produced a 60 Hz magnetic field of 0.05 G at the center of the chamber. These fans were replaced by Rotron, Inc., "Feather" fans with hysteresis motors, which produced about 0.003 G

at the same position as measured by a Hall probe. Heater wires installed to prevent frost on the door gaskets were disconnected.

The mitosis time of *Physarum* is dependent on temperature. Three incubators were originally equipped with electronic temperature control units using thermistor sensors inside the chambers; the oldest unit was refitted with an identical control. To prevent the temperatures in the individual incubators from slowly drifting away from each other, three of the controllers were modified to sense and act to eliminate differences in temperature between that incubator's interior and the unmodified controller's chamber.* The standard controller circuit is a resistance bridge in which the potential drop across the thermistor inside the incubator is compared to the drop across a control potentiometer (1). The modification replaced the control potentiometer with a second thermistor located in the "master" incubator and changed some of the fixed resistors in the circuit to make the currents through both thermistors equal.

One incubator therefore had the "master" temperature control, the others were "slaved" to the first. The master control was located in the incubator housing our control cultures. The four chamber temperatures were adjusted to be equal to within 0.15°C ; they did not drift beyond a difference of 0.3°C . The normal operating temperature was $26.0 \pm 0.3^{\circ}\text{C}$.

Temperatures were monitored continuously by Rustrak strip-chart recorders reading a separate thermistor in each incubator. Figure 1 shows a sample of the temperature records. Temperature recorder calibration was checked daily against a mercury-in-glass thermometer mounted inside each incubator. Temperatures at various points in the experimental regions

* We are indebted to Mr. Mel Beard of Waynco, Inc., Winona, Minn. for supplying circuits and information which enabled us to make this alteration easily.

generally were within 0.3°C of the nominal value, but a few points departed by up to 0.5°C .

The interior shelving of the incubators was replaced by a structure carrying the magnetic field coils and a shelf for the cultures of macroplasmidia grown on filter paper. The shelf was constructed of Masonite "pegboard" to permit more uniform air circulation within the chamber. A New Brunswick Scientific Co. Model RS-8 reciprocating shaker was placed on the floor of each incubator and was adjusted to a rate of 122 cycles per minute. Extension posts of aluminum carried the moving shelf for the submerged cultures of microplasmidia into the center of the magnetic field coils. Figure 2 shows the arrangement of the interior of the incubator. The internal structure was constructed of non-magnetic materials to avoid concentration of the magnetic flux. Four large steel screws holding the moving shelf to the aluminum posts were in the neighborhood of the submerged cultures, however. The shaker and the incubator walls were the main steel components; both were located outside the magnetic coils.

We measured the vibrations experienced by cultures on both the stationary and shaken shelves with a Columbia Model 902 D.C. Piezoelectric accelerometer. The spectra were qualitatively very similar in all incubators, displaying a 60 Hz component correlated with the refrigeration compressor of the incubator and a component at about 1 kHz correlated with the shaker. The control incubator had a slightly lower amplitude spectrum than the others. Figure 3 shows oscilloscope traces of the accelerometer output.

III. Magnetic Fields

The magnetic fields were generated by passing current through parallel rectangular coils connected in series; each coil was wound with 50 turns of #16 enameled copper magnet wire. The coils were approximately 63×85 cm and were 37 cm apart. The four coils in series had a DC resistance of

about 8 ohms and an impedance at 75 Hz of about 13 ohms. They produced a field strength of about 1 G/A.

The units were designed to produce magnetic fields uniform to $\pm 10\%$ over the region of use. This target was met, as can be seen in the two representative field plots shown in Figures 3 and 4, which were made using a F. W. Bell Model 640 Hall probe gaussmeter. Field plots such as these were made for each incubator on a 5 cm square grid at both the stationary and shaker culture shelves. The 60 Hz field from all causes, including the incubator and shaker, was less than 0.01 G. Plots were made whenever field conditions were changed, and spot-checks of magnetic field values at six representative points in each incubator were made every 2-4 weeks. An oscilloscope check of the sinusoidal waveform of the Hall effect signal was also made.

IV. Electric Fields and Growth Flasks

To produce a uniform electric field across *Physarum* grown in a nutritive medium which is electrically polarizable, we chose to place the medium in electrical contact with two parallel plane electrodes. The medium has a resistivity of about 200 ohm-cm at 75 Hz. Direct current is blocked by a capacitor, although about 0.15 mA of alternating current flows through each cell. Cells were designed so the desired field intensity could be produced by application of low potentials (≤ 40 mV), thereby avoiding complications due to electrolysis.

Conventionally, *Physarum* microplasmodia are grown in Erlenmeyer flasks with an antiseptic stopper. Macroplasmodia are cultured in Petri dishes on Millipore filters or filter paper supported on stainless steel grids at the surface of the medium (2, 3). We needed a rectangular flask to provide a uniform field throughout the region in which the microplasmodia grow. Our flasks had to be non-magnetic and had to withstand

repeated trips through the laboratory dishwasher and autoclave. The successful design used glass, plastics, Type 304 stainless steel, and General Electric RTV-118 silicone rubber adhesive-sealant. Ours is the first use of the latter material with Physarum to our knowledge.

The flask for the cultures of microplasmodia is shown in Figure 6. The two long walls are stainless steel; the remaining walls and bottom are $\frac{1}{4}$ in. (0.63 cm) plate glass. The top is $\frac{1}{4}$ inch General Electric Lexan polycarbonate plastic which was chosen in place of the less-expensive glass so the large hole could be machined more easily. The hole is closed with a polyurethane foam stopper. All pieces are thoroughly degreased, cleaned in a hot chromic acid solution, and rinsed thoroughly before the silicone rubber joints are made. The edge of the plastic is roughened with a hot soldering iron to help the silicone rubber sealant adhere.

Electrical contact is made from banana jacks in the shaker table to the electrodes through plugs soldered to Teflon-insulated #22 stranded copper wire. The other end of the copper lead is brazed to a stainless steel tab, which is spot-welded onto the electrode. The wire is bound to the tab and plug to relieve strain, and a brass sleeve is soldered onto the plugs to serve as a handle, discouraging removing the plugs by pulling on the wires. The jacks in the shelf are connected by a cable to control panels outside the incubator, as explained below.

The flasks contain 23 ml of nutritive fluid medium (4), which fills them to a depth of about 1 cm. Under typical experimental conditions about 0.15 mA of AC current flows at 75 Hz when 35mV is applied across the cell, producing an electric field of 0.7 V/m. The potential across the cell is modulated slightly by the action of the shaker, which alters

the contact area between the liquid and the electrodes as it moves, changing the cell's impedance slightly. The field modulation is less than $\pm 5\%$; an oscilloscope trace of the modulation is shown in Figure 7. The motion of the shaker was perpendicular to the electric field direction and parallel to the plane of the electrodes.

Electric fields were applied to the macroplasmodia with stainless steel electrodes fastened to the lids of the Petri dishes. The modified Petri dish is shown in Figure 8. A piece of $\frac{1}{4}$ in. thick cellulose milk filter supported a piece of filter paper at the surface of the nutritive medium; the stainless steel grids normally used for supports could not be used because of their electrical conductivity. Stainless steel electrodes were spot-welded onto 0.125 in. diameter stainless steel wire, which passed through two small holes drilled in the Petri dish lid. Silicone rubber sealant held the metal assemblies in place and sealed the holes. Standard tip-jacks fit over the stainless wires and make electrical connections through insulated copper wires to banana jacks on the stationary shelf of the incubator.

A plot of the potentials across a piece of filter paper is shown in Figure 9. The electric field, given by the change in potential per unit distance, is uniform to far better than the $\pm 10\%$ target. The potential readings were made by applying 33 mV across an open-top plate and using a Hewlett-Packard Model 427A AC voltmeter to measure the potential between one electrode and a thin copper probe carried on a micromanipulator. The probe was the center conductor of a coaxial cable, and care was taken to avoid ground loops and other sources of spurious signals. In another plot made using filter paper with a plasmodium growing on it, no difference in the field uniformity was seen

between points inside and outside the area covered by the plasmodium. We did not observe the probe microscopically to see whether the probe was inside the plasmodium or had penetrated through to the filter paper beneath.

In operation, the vessels are placed in position, the electrical leads are connected to the jacks in the shelving, and a potentiometer is used to adjust the potential across the vessel, as measured by a Hewlett-Packard Model 400F AC voltmeter. The potential across any one shaker flask and any one Petri dish in each incubator is continuously recorded. Checks of the sinusoidal shape of the potential waveform and of the relative phase of the electric and magnetic fields were made at the same time as the checks of the magnetic field values.

In the course of the experiment it was noticed that a small DC potential regularly appears across the electrodes of both the shaker flasks and the Petri dishes in each incubator, including the one housing control cultures. Both polarity of the potentials and its magnitude depend on the individual vessel. In measurements on a number of vessels the polarity reverses randomly and the magnitude of the potential will range from 1 or 2 mV up to about 25 mV. Direct current through the vessels is normally blocked by an external capacitor; if current is allowed to flow, one finds that the vessel itself is the seat of the potential and that it has a source impedance of several megohms. Outside the incubators the vessels and even isolated pairs of samples of electrode material inserted into beakers of distilled water displays similar behavior. The potentials probably originate in some asymmetries in the metal-electrolyte interfaces; similar effects have been reported by others, for example by Lykken (4) and Greenwald (5).

V. Electrical Circuits, Monitoring, and Alarm System

Each of the three incubators containing cultures to which fields are being applied has its own set of power supplies and monitoring equipment. Sinusoidal signals at 60 or 75 Hz are generated by a Wavetek Model 130 oscillator and are amplified by an Elgar Model 201 or 501 Power Amplifier. A schematic circuit diagram is shown in Figure 10. The magnetic field coils and the electric field circuits are connected to the amplifier in parallel. Each set of banana jacks on the shaker or stationary shelves inside the incubator is wired to its own control potentiometer and blocking capacitor, mounted on control panels on a relay rack outside the incubator. All equipment for a given incubator is run from line power taken from a separate isolation transformer. Care is taken to avoid spurious signals by using a single common point located inside the incubator, as shown on the schematic. The common point is not grounded. The cable linking the control panels to the incubators is six-pair microphone cable with each pair individually shielded. The wires of five pairs feed power to ten stations on one shelf. One lead of the sixth pair returns the common to the relay rack, and the unused lead and each shield are connected to common inside the incubator. The relative phase of the two fields is adjusted by a series capacitor in the electric field-supply branch of the circuit. The control incubator is wired in the same manner as the other three, except that the power amplifier is replaced by a five ohm resistor, representing the impedance of the amplifier.

As noted earlier, several parameters are continuously recorded by Rustrak miniature strip-chart recorders. These parameters are the four incubator temperatures, the three magnetic coil currents, and representative shaker flask and Petri dish potentials from the three incubators

in which applied fields are present.

A battery-operated alarm system was built to warn the experimental team of failures which would endanger the survival of the cultures or interrupt the continuity of the exposure. The circuit is shown in Figure 11. A standard telephone company teletypewriter line links the laboratory with the campus heating plant, which is manned continuously. A signal to notify the team is activated whenever an incubator temperature is above or below preset limits or power output from one of the Elgar amplifiers ceases. A separate relay removes power from the shaker inside an incubator which has exceeded its temperature limits, removing the main source of heat and preventing further temperature rises. The shaken cultures can survive about two hours without further aeration, giving the team sufficient time to make adjustments. Auxiliary power is available during failures of the main laboratory electrical supply.

VI. Operating Experience and Conclusions

The equipment has proved quite reliable in operation. No major difficulty in its use or deterioration in performance has been noticed since the first unit was activated in mid-1971. The modified shaker flasks and Petri dishes have proved as satisfactory as the more traditional methods of culturing *Physarum*.

Data and the results of our experiments using the equipment are presented above.

VII. Acknowledgements

The authors wish to thank Mr. James Lubniewski and Mr. Thomas Lubniewski, student assistants, for their willing and skilled help in assembling and maintaining the equipment and for their design suggestions.

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FIGURE CAPTIONS

1. Traces from temperature recorders, showing absolute and relative stability of temperatures in each incubator.
2. General view of the interior of the incubators. All four incubators had identical interior fittings. The coordinate system indicated has its origin in the upper right rear corner of the incubator. It is used to specify location within the incubator when plotting field intensities.
3. Oscilloscope traces from a piezoelectric accelerometer mounted near the end of the stationary or moving shelf, as indicated in the figure. In each photograph the vertical scale is 0.005 V/division, representing an acceleration of approximately 0.3 g's/cm. The horizontal scales for each photograph's upper trace is 0.1 sec/div; for the lower traces the horizontal scale is 0.02 sec/div.
4. Magnetic field data (in Gauss) as measured by the Hall-effect gauss-meter. Each number represents the AC magnetic field strength on a 10 cm grid. The distances are measured using the coordinates defined in figure 2.
 - a) Fields in the plane of the stationary shelf
 - b) Fields in the plane of the shaker shelf
5. Trends in the magnetic field data given in figure 4.
 - a) Variation in stationary shelf field strength with position in the direction of the field (z), plotted for various distances (X) from the rear of the incubator.
 - b) Variations in shaker shelf magnetic field strength with position in the direction of the field (z).
 - c) Variations in magnetic field with height (y).

(FIGURE CAPTIONS - continued)

6. Rectangular shaker flask using parallel plane electrodes as two sides of the vessel.
7. Variation in potential across a shaken flask due to shaking motion. The shaker is making 122 oscillations per minute; the electrical signal is a 75 Hz sine wave.
8. Modified Petri dish containing filter paper and macroplasmodium.
9. Electric potential data across filter paper in a Petri dish. The data show measured potential as a percentage of applied potential, which was a 33 mV (rms) 75 Hz sine wave.
 - a) Potentials plotted on a 1/8 in. grid (.32 cm).
 - b) Plot of potentials in columns labeled "A" and "B" in figure 8a. The slope of the graph at any point is equal to the electric field strength there; a straight-line graph indicates a uniform field.
10. Schematic diagram of circuits producing electric and magnetic fields. As explained in the text, the control incubator was also wired in this way, but had a resistor connected in place of the amplifier-oscillator chain.
11. Schematic diagram of alarm circuit.

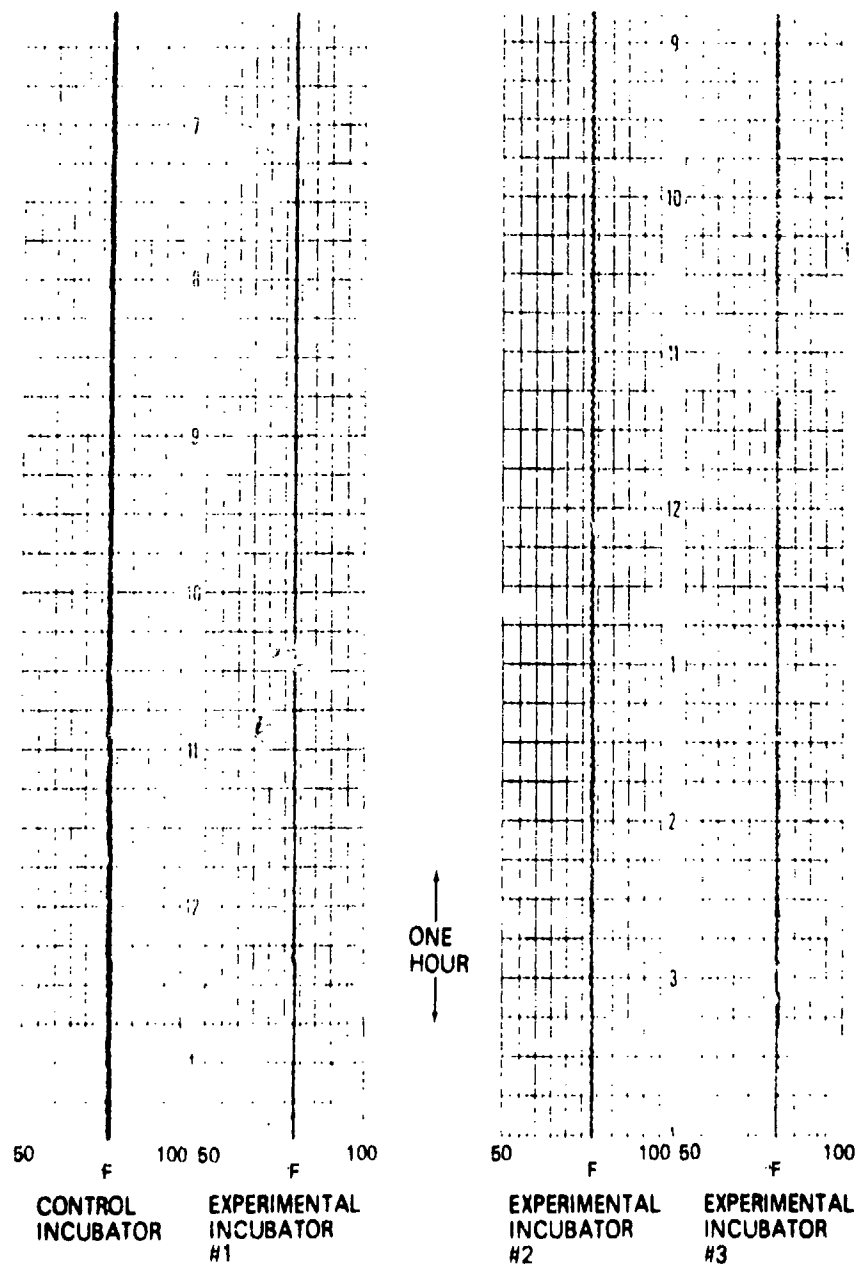


Figure 1

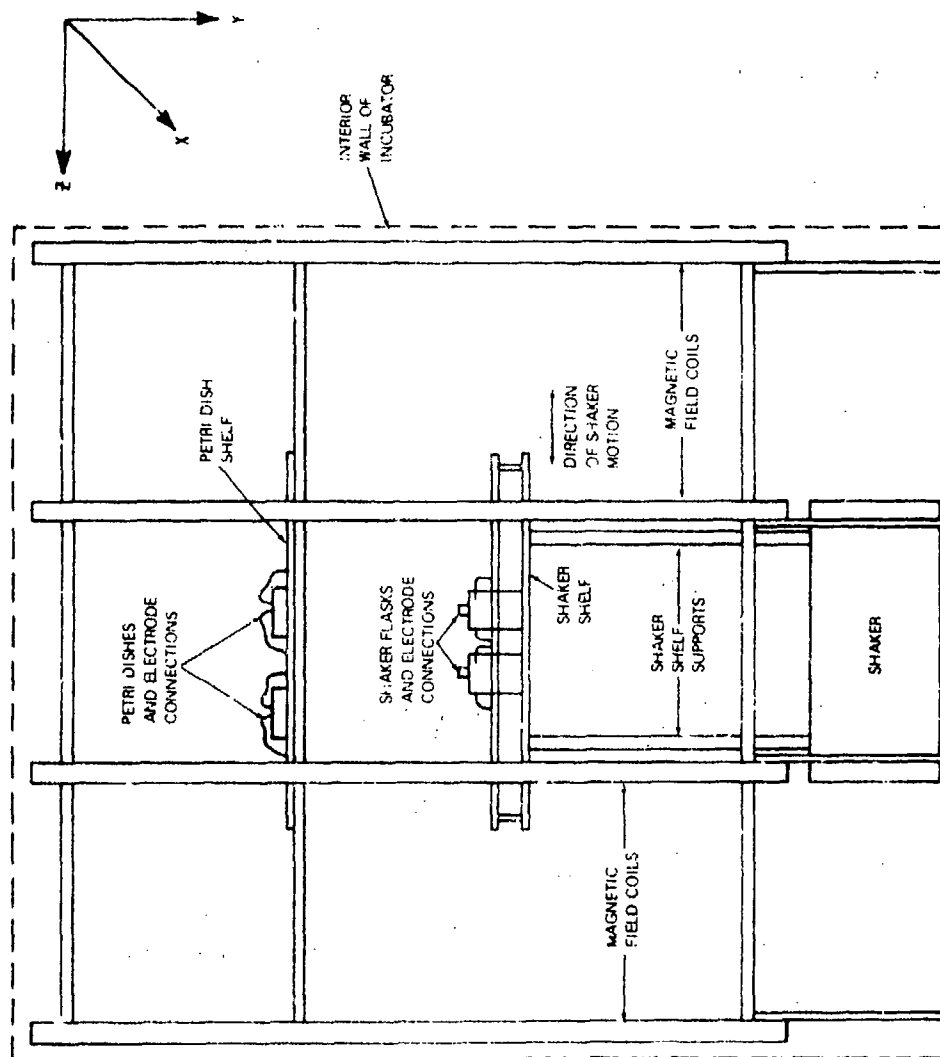
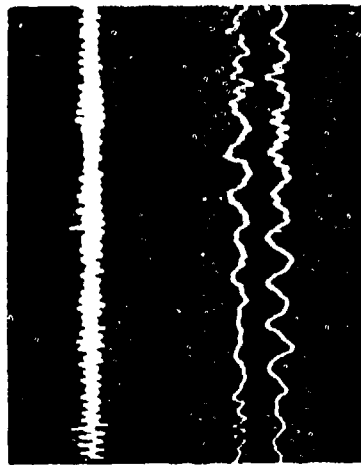
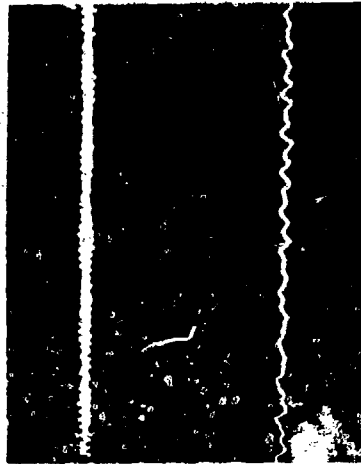


Figure 2

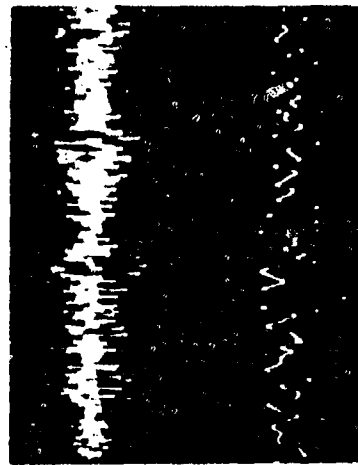
0.1 SEC



0.02 SEC
PETRI DISH SHELF
CONTROL INCUBATOR



PETRI DISH SHELF
EXPERIMENTAL INCUBATOR #1
(2.0G 0.7V M)



SHAKER SHELF
CONTROL INCUBATOR



SHAKER SHELF
EXPERIMENTAL INCUBATOR #1

Figure 3

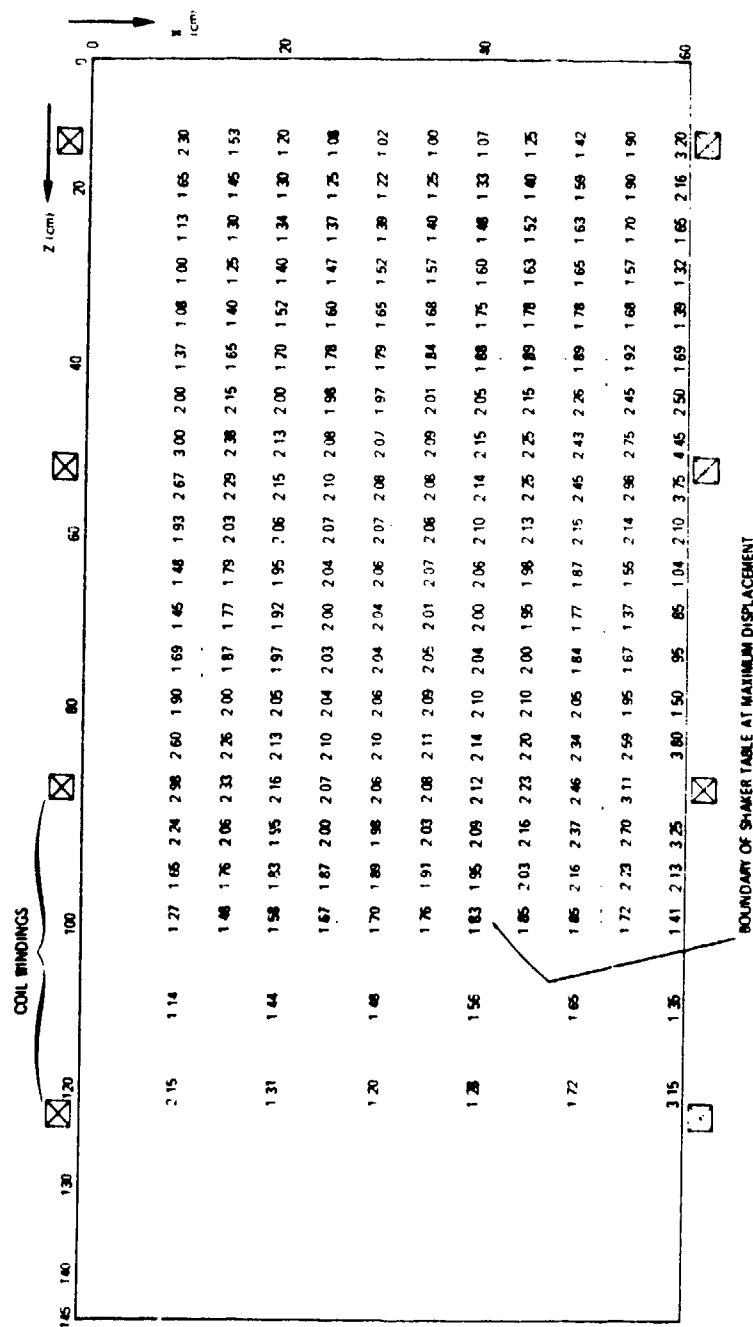


Figure 4b

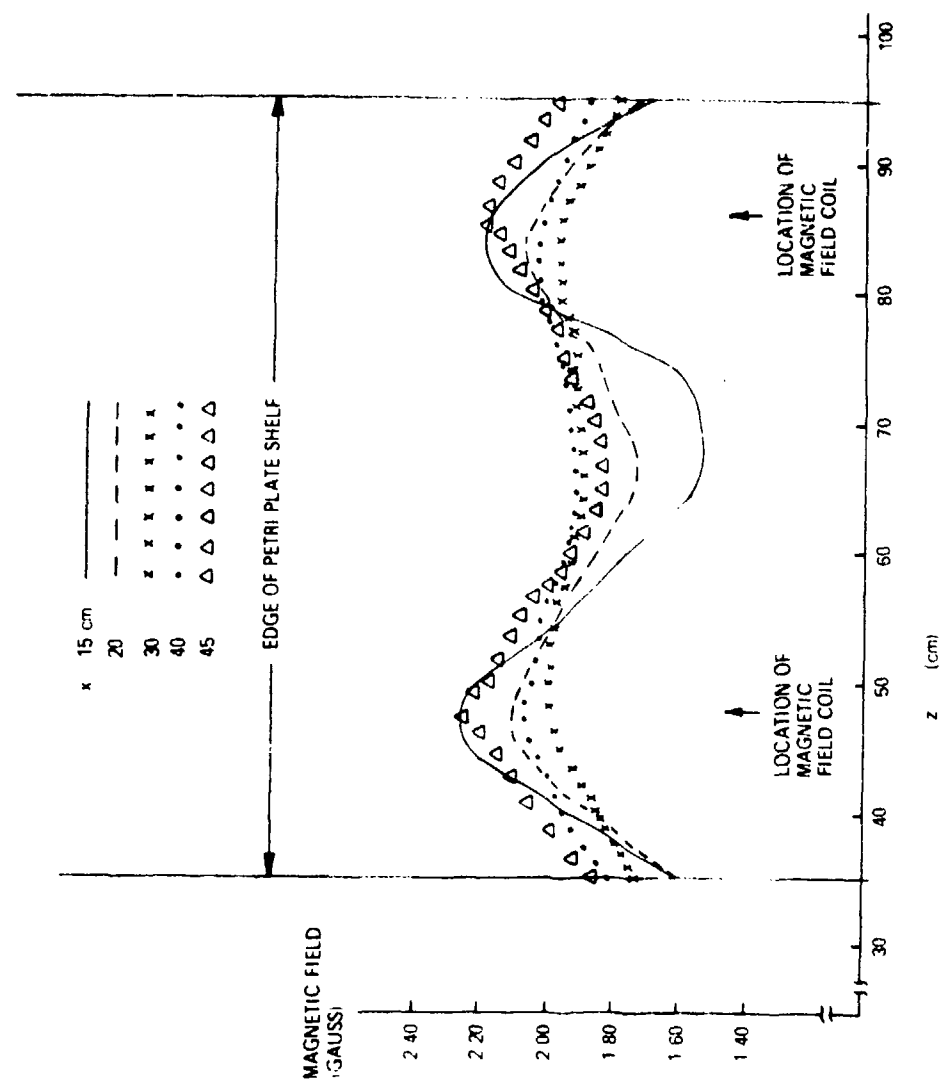


Figure 5a

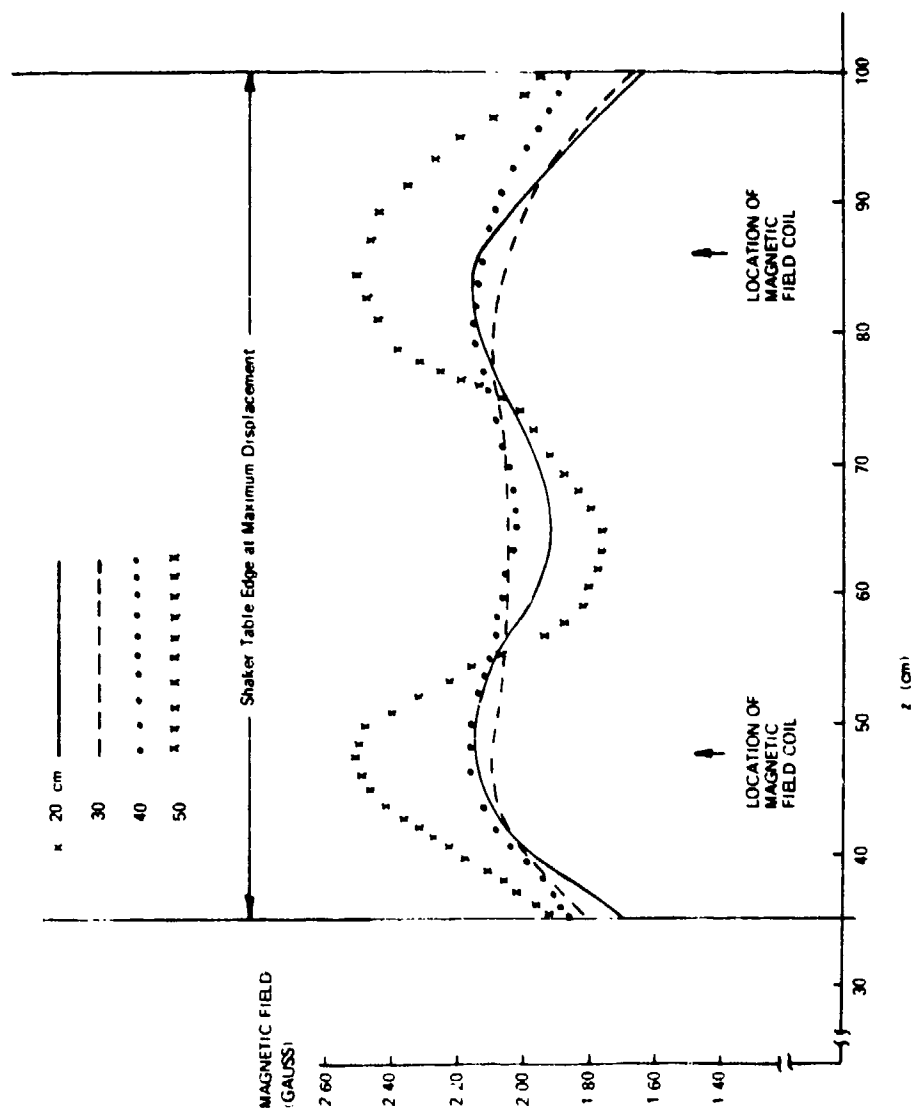


Figure 5b

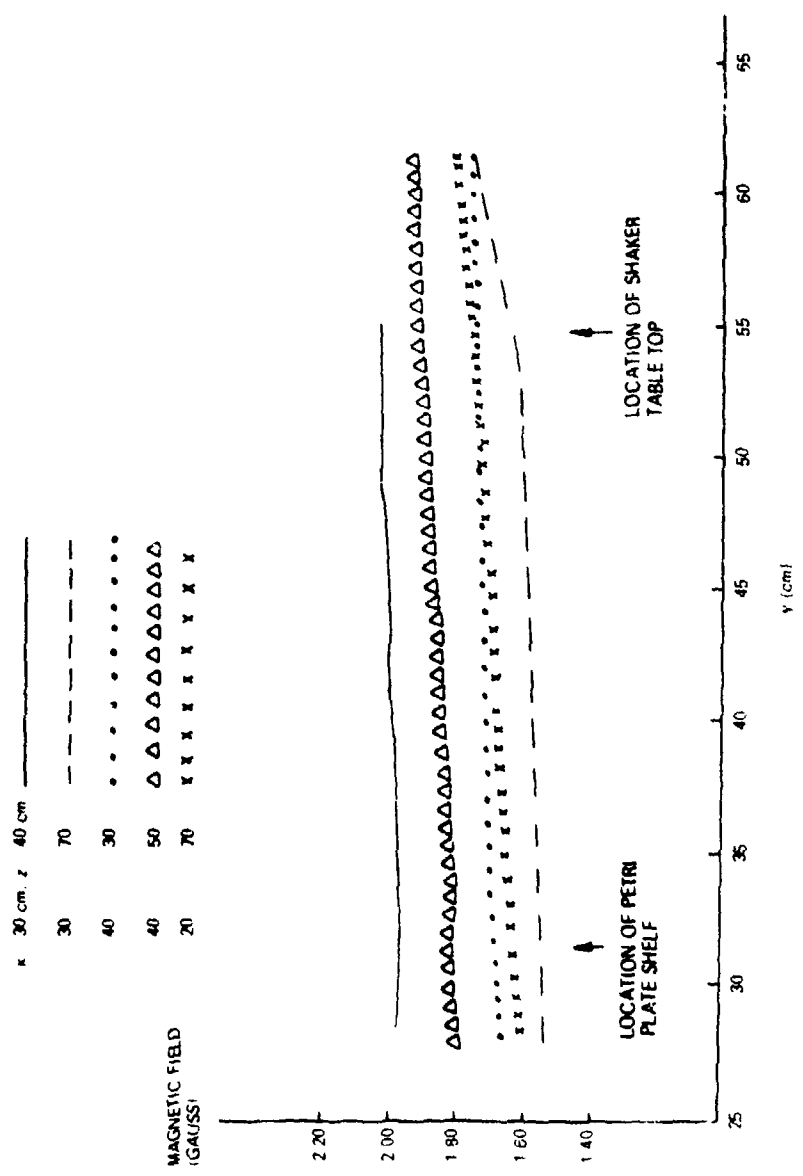


Figure 5c

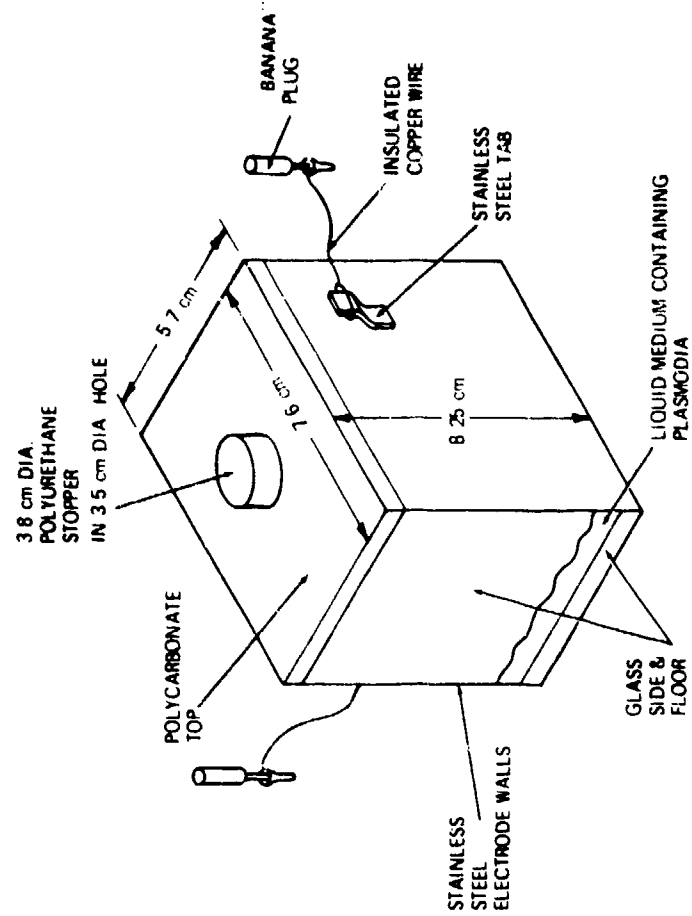
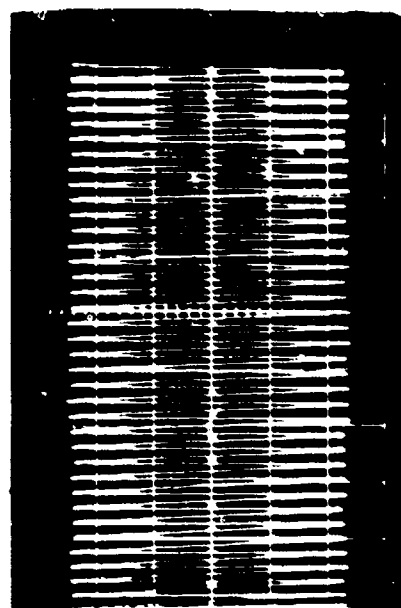


Figure 6



0.02 V

0.05 SEC

Figure 7

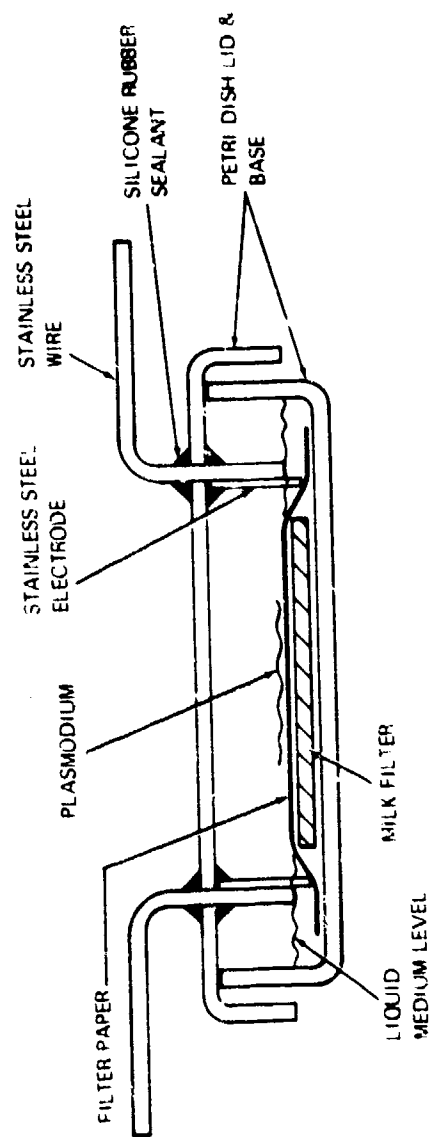


Figure 3

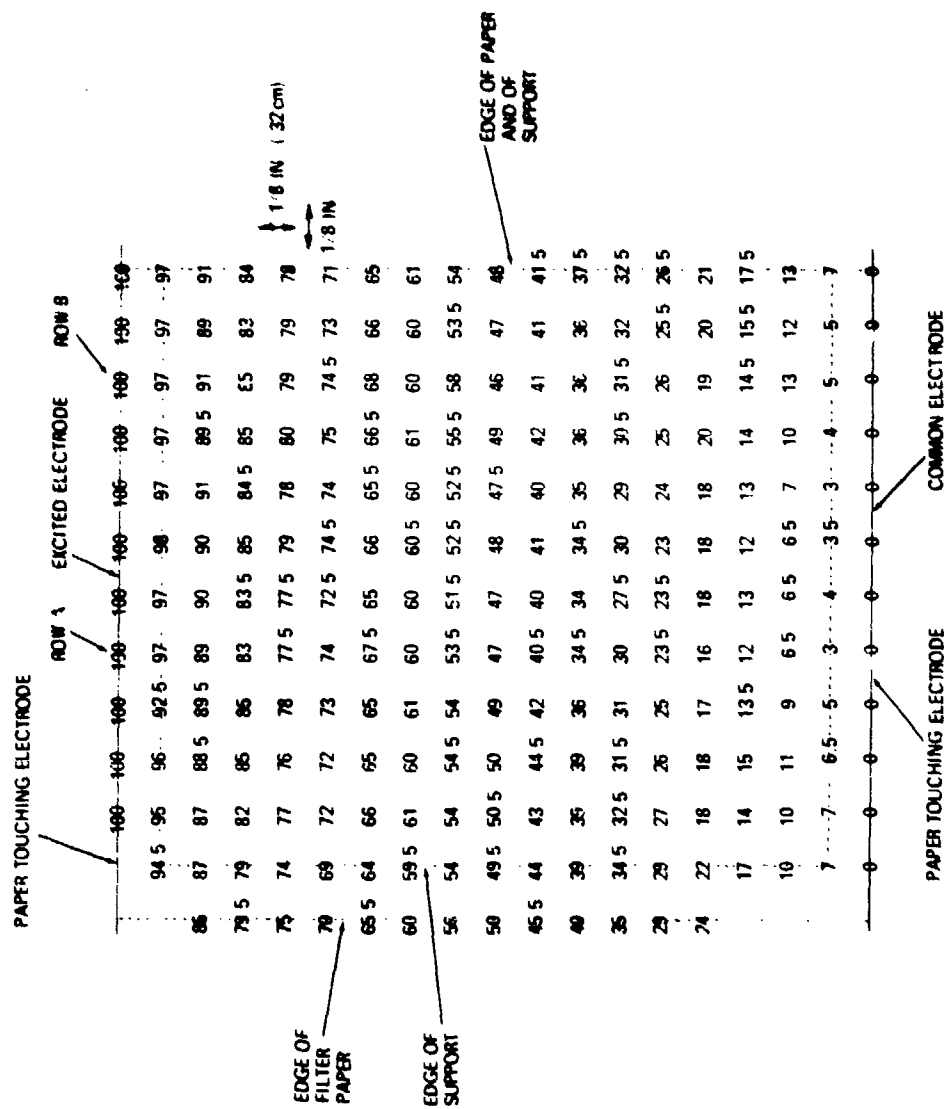


Figure 9a

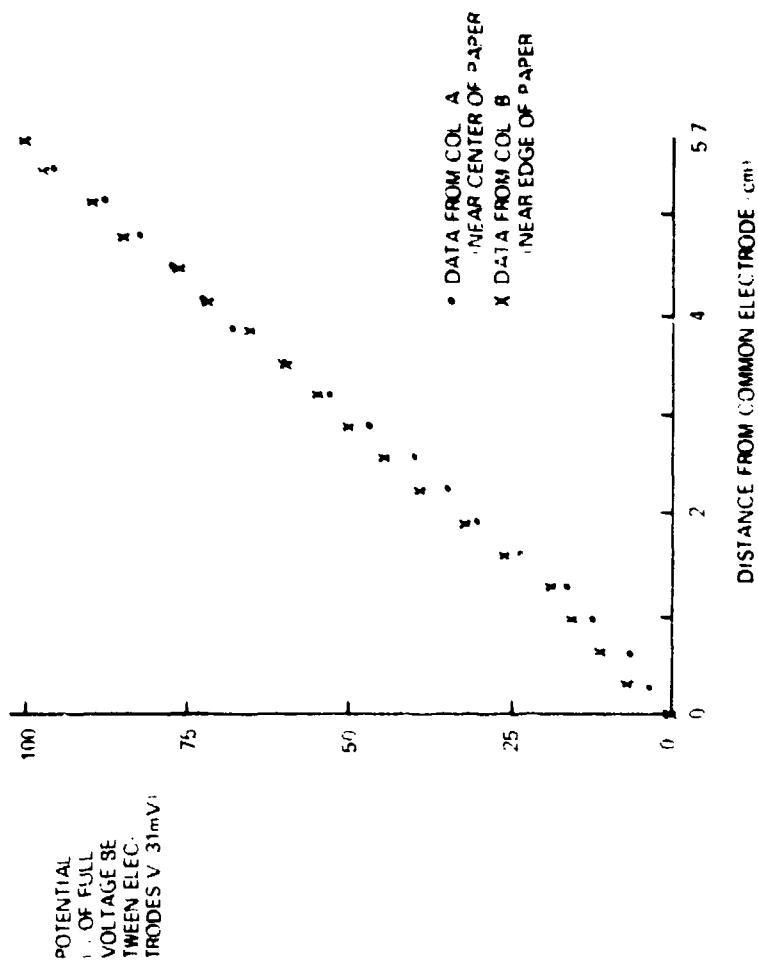


Figure 91

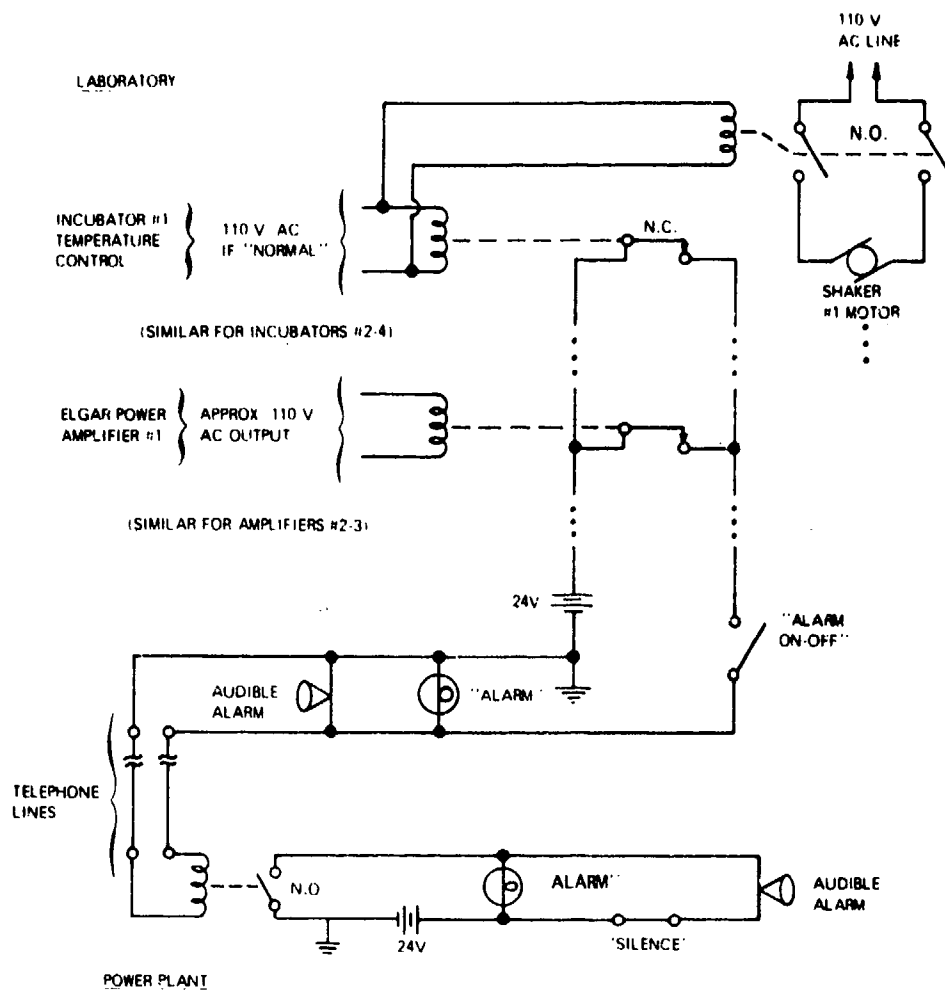


Figure 11